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Increased Src kinase level results in increased protein tyrosine phosphorylation in scrapie-infected neuronal cell lines

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Abstract **We have studied how prion infection may affect the Src kinase activity in three different neuronal cell lines, ScGT1 and ScN2a, where ScGT1 were generated in our laboratory. By immunoblotting, using clone 28 – a monoclonal antibody recognizing active Src, we have found a 32 ± 6.3% and 75 ± 7.7% elevation in Src activity in ScGT1 and ScN2a cells, respectively, compared to uninfected cells. Immunocomplex in vitro kinase assay confirmed the increased Src activity. The increased Src kinase activity in scrapie-infected cells was further shown to correlate to an increased level of Src protein.**

In addition, an important increase in the protein tyrosine phosphorylation signal was observed in ScGT1 and ScN2a cells, which was further shown to be Src-dependent, as treatment with PP2 – a Src family kinase specific inhibitor, reversed the protein tyrosine phosphorylation profile. Abnormal Src-kinase activation and subsequent protein tyrosine phosphorylation may be key elements in the neuropathology of the prion diseases.
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1. Introduction

The cellular prion protein (PrP^C) is a ubiquitous glycoprotein, mainly expressed in neurons. In prion diseases PrP^C is converted into an abnormal conformer, designated pathogenic scrapie isoform of PrP^C (PrP^{Sc}), enriched in β-sheets and resistant to proteinase K (PK) digestion [1]. PrP^C is a glycosylphosphatidylinositol (GPI)-anchored protein, predominantly distributed in synapses [2] and localized to sphingolipid- and cholesterol-rich membrane domains also called lipid rafts [3]. Lipid rafts are highly specialized microdomains, functioning as platforms for the attachment of lipid-modified proteins e.g. GPI-anchored proteins, receptor tyrosine kinases (RTKs) and Src-family tyrosine kinases. Lipid rafts and early endosomes are also suggested to be the site of conversion of PrP^C to PrP^{Sc} [4].

Although the physiological function of PrP^C still remains unclear, several plausible roles of PrP^C have emerged. One suggests that PrP^C protects against oxidative stress, possibly through the regulation of synaptic copper transport [5]. Another imply PrP^C as a cell-surface receptor, mediating neuronal survival [6] and neurite outgrowth, as shown by laminin-induced neuritogenesis of primary neurons from wild-type but not PrP null mice [7]. Consistent with a role of PrP^C as a signaling molecule is the finding that (I) PrP^C co-localizes with and co-immunoprecipitates Src-family kinases [8] and (II) antibody-mediated ligation of PrP^C promotes dephosphorylation and activation of Src-family kinases in various cell types [9,10]. In mice devoid of PrP^C, long-term potentiation is impaired, indicating a synaptic function of PrP^C [11]. Synaptic degeneration is one of the earliest neuropathological features in prion diseases [12].

Src is the prototype member of a family of non-receptor protein tyrosine kinases with nine members, five of which Src, Fyn, Lyn, Lck and Yes are expressed in the central nervous system (CNS). Within CNS neurons, Src is found both pre- and post-synaptically [13]. Src-family kinases control diverse cellular functions such as cell proliferation, differentiation and apoptosis [14]. In the adult nervous system, Src kinases are implicated in ion channel activity and synaptic plasticity, as well as in neurodegeneration [15].

Given that PrP^C both colocalizes with and may activate Src-family kinases, we hypothesized that the formation and accumulation of PrP^{Sc} in raft-domains may induce an uncontrolled activation of Src-kinases, possibly responsible for the synaptic degeneration in prion diseases. This study therefore sought to identify how prion-infection may affect the activity of the Src-kinase as well as its downstream signaling targets.

2. Materials and methods

2.1. Materials

Anti-PrP (sc-7694) and N-16 (sc-19) antibodies were purchased from Santa Cruz Biotechnology Inc. HRP-conjugated sheep-anti-mouse and protein-G sepharose beads (4FF) were from Amersham-Biosciences. Anti-phosphotyrosine (anti-pTyr) antibody (4G10) was from Upstate Biotechnology Inc. Clone 28 antibody [16] was kindly provided by Prof. Koji Owada at Kyoto Pharmaceutical University, Japan. Mouse monoclonal pp60Src antibody (clone 327) [17] was a kind gift of Prof. Joan Brugge, Harvard Medical School, USA. 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo(19)pyrimidine (PP2) was purchased from Calbiochem. [γ-^{32}P]ATP, 10 Ci/mmol was from Perkin–Elmer Life and Analytical Sciences, Boston, MA. All cell culture reagents were from Invitrogen, Stockholm, Sweden. All other reagents were from Sigma.

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Abbreviations: CNS, central nervous system; GPI, glycosylphosphatidylinositol; IP, immunoprecipitate; mAb, monoclonal antibody; PBS, phosphate buffered saline; PK, proteinase K; PP2, 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo(19)pyrimidine; PrP^C, cellular prion protein; PrP^{Sc}, pathogenic scrapie isoform of PrP^C; pTyr, phosphotyrosine; RTKs, receptor tyrosine kinases; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis

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2.2. Generation of ScGT1 cells

Brain homogenate from Rocky Mountain Laboratories (RML) prion-infected CD-1 mice was kindly provided by Prof. Stanley B. Prusiner at UCSF, CA. ScGT1a and ScGT1b cells were generated at two different occasions by inoculating GT1-1 [18] cells (immortalized murine hypothalamic neuronal cell line) with RML brain homogenate as described in [19]. ScN2a cells were together with the non-infected murine neuroblastoma cell line N2a generously provided by Stanley B. Prusiner. N2a cells were also purchased from ATCC.

2.3. Cell culture and detection of PrP^{Sc}

All cells were routinely cultured at 37 °C under an atmosphere of 5% CO_2 in DMEM with Glutamax II and 4.5 g/l D-glucose supplemented with penicillin–streptomycin and 5% fetal bovine serum (N2a/ScN2a) or 5% fetal bovine serum and 5% horse serum (GT1/ScGT1a/b). ScN2a, ScGT1a and ScGT1b were regularly controlled for scrapie infection by detection of PrP^{Sc}, as described in [19].

2.4. PP2 treatment

2×10^6 GT1/ScGT1b cells or 5×10^5 N2a/ScN2a cells were seeded in 6 cm culture dishes three days before treatment for 24 h in the presence or absence of 1 μM PP2 for N2a/ScN2a or 3 μM PP2 for GT1/ScGT1. A lower concentration of PP2 (1 μM) was used for N2a/ScN2a cells because 3 μM PP2 was lethal to the N2a cells. After treatment, cell lysates were subjected to anti-pTyr immunoprecipitation and/or anti-pTyr immunoblotting. Western blot and immunoprecipitation were performed as described in [20].

2.5. In vitro Src kinase assay

1 mg of protein from the indicated cell lysates was immunoprecipitated with the Src-antibody (N-16). Following immunoprecipitation the protein-G sepharose beads were washed three times with kinase buffer (50 mM HEPES, 10 mM $MgCl_2$, and 1 mM $MnCl_2$). The in vitro kinase reaction was carried out in 20 μl kinase buffer containing 3 μM unlabeled ATP, 3 μCi of [γ-^{32}P]ATP, 10 μg of the exogenous Src kinase substrate muscle rabbit enolase (Sigma E0379). The enolase was pretreated in 25 μM acetic acid for 10 min at 30 °C. Samples were incubated for 30 min, 30 °C and stopped by the addition of 10 μl of 4× Laemmli sample buffer and boiled for 5 min. 10 μl of the reaction mix was separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The gel was dried and exposed to a X-ray film for 18 h. Densitometric measurements of the autoradiographic results was performed on a PC using the program Image Gauge, v. 3.46.

3. Results

3.1. Generation of scrapie-infected GT1 cells (ScGT1a and ScGT1b)

In addition to ScN2a cells, two scrapie-infected GT1 cell lines (ScGT1a and ScGT1b) were generated by inoculating the highly differentiated hypothalamic GT1 cell line with RML-brain homogenate, with the aim to obtain several prion-infected cell lines. The presence of PrP^{Sc} was analyzed after six passages (2 months) by immunoblotting of PK-treated cell lysates with a PrP specific antibody. PK-resistant PrP^{Sc} was detected in ScN2a, ScGT1a and ScGT1b, but not in uninfected N2a and GT1 cells (Fig. 1). ScGT1b cells showed a ∼3-fold increased level of PK-resistant PrP^{Sc} compared to ScGT1a. At present we cannot distinguish if a larger proportion of cells in the ScGT1b population is infected (i.e. produce PrP^{Sc}) or if certain individual cells in ScGT1b produce an increased amount of PrP^{Sc}, which on a Western blot of PK-treated cell lysate would give the same result.

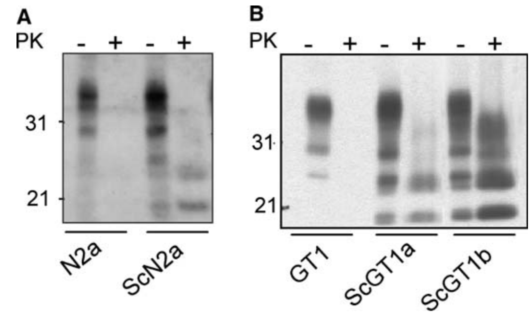

Fig. 1. Detection of PK-resistant PrP^{Sc} in ScN2a and ScGT1a/b cells. Non-treated (−) and PK-treated (+) cell lysates of N2a, ScN2a, GT1 and ScGT1a/b were loaded on 12% polyacrylamide gel, transferred to nitrocellulose membrane and PrP isoforms were detected by the specific anti-PrP antibody. 90% of each cell lysate (0.45 mg) was PK-treated with 20 μg PK/mg protein for 1 h at 37 °C. Remaining 10% of the cell lysate was undigested.

3.2. Increased levels of active Src kinase in scrapie-infected cells

The level of active Src was determined by Western blot of immunoprecipitated Src with monoclonal antibody (mAb) clone 327 and further blotted with mAb, clone 28 (recognizing active Src), Fig. 2A. Densitometric measurements revealed a 32 ± 6.3% ($P = 0.0418$, $n = 3$) increase in clone 28-immunoreactivity in ScGT1b compared to GT1 cells (Fig. 2A) when the same amount of protein was loaded. A significant increase was also observed in ScN2a, i.e. 75 ± 7.7% ($P = 0.0168$, $n = 3$) increase compared to uninfected controls (Fig. 2A). The blots in Fig. 2A were dehybridized and rehybridized with the Src antibody clone 327 in order to determine the protein level of Src (Fig. 2B). Comparing the amount of active Src (clone 28-immunoreactivity, Fig. 2A) captured in the clone 327-IP, to the amount of total Src in the same lanes (clone 327-immunoreactivity, Fig. 2B), no increase in the specific activity of Src was observed. A similar increase in Src protein level (25%) was also found in the independently prion-infected ScGT1a (Fig. 2D), strongly indicating that increased Src protein level is a result of scrapie infection and not a clonal effect, as an increased amount of Src can be shown in several RML-infected neuronal cell lines.

3.3. Increased tyrosine phosphorylation in scrapie-infected cells

To determine whether the increased levels of active Src in scrapie-infected cells could influence the overall protein tyrosine phosphorylation of cellular proteins, anti-pTyr staining of whole cell lysates was performed. As shown in Fig. 3A, a major change in pTyr profile was observed between GT1 and ScGT1b, with an increased phosphorylation of several high molecular weight protein bands of 70–180 kDa in ScGT1b (Fig. 3A). Similar changes were also observed comparing N2a and ScN2a cells, with both the magnitude and the number of tyrosine phosphorylated proteins increased compared to N2a. In both ScGT1b and ScN2a cells the majority of proteins with increased tyrosine phosphorylation is phosphorylated also in the native cells, although to a much lesser extent. Still, two proteins (≈pp97 and ≈pp180) only appeared in ScN2a, indicating also novel targets for the increased tyrosine phosphorylation in ScN2a. The overall tyrosine phosphorylation in ScGT1b and ScN2a were 71 ± 8.1% ($P = 0.032$, $n = 3$) and

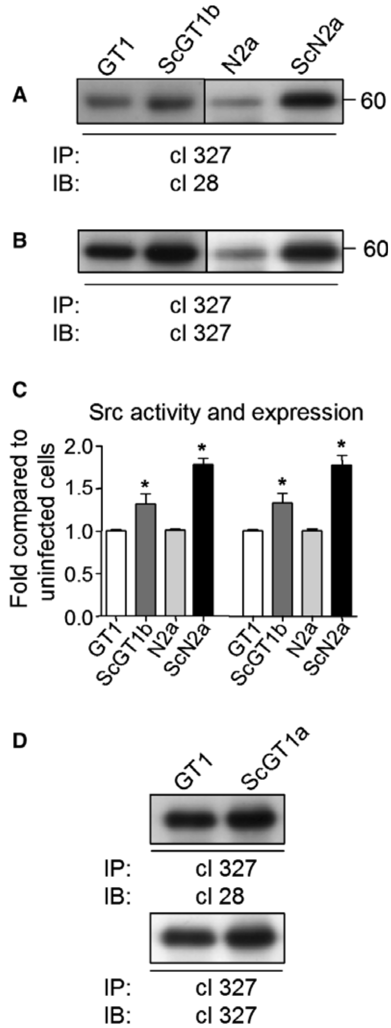

Fig. 2. Increased levels of active Src protein levels in scrapie-infected cells. (A) Anti-Src immunoprecipitates (clone 327-IPs), from 1 mg of protein from the indicated cell types were immunoblotted with clone 28. (B) The blots were dehybridized and rehybridized with clone 327. (C) Densitometric measurements of (A) and (B), respectively. (D) Anti-Src immunoprecipitates (clone 327-IPs) from 1 mg of protein from GT1 and ScGT1a were immunoblotted with clone 28 (upper panel). The membrane was dehybridized and rehybridized with clone 327. IP = immunoprecipitated, IB = immunoblotted, cl 327 = clone 327, * = $P < 0.05$, Student's *t*-test, $n = 3$.

226 ± 21% ($P = 0.047$, $n = 3$) elevated compared to GT1 and N2a, respectively, as assessed by densitometry.

A higher resolution of the scrapie-induced increase of phosphorylated proteins was obtained by pTyr-staining of anti-pTyr immunoprecipitates from uninfected and infected cells (Fig. 3B). Three major bands with an increased tyrosine phosphorylation were detected in ScGT1b and ScN2a, although of different molecular weights, possibly with the exception of the ≈pp116 protein. All of them were mainly or completely absent in immunoprecipitates from GT1 and N2a, respectively. Densitometric measurements showed a ≈4-fold enrichment in tyrosine phosphorylated proteins in ScGT1b (4.13 ± 0.4, $P = 0.002$, $n = 3$) and ScN2a (4.4 ± 0.5, $P = 0.006$, $n = 3$), respectively, compared to uninfected cells. A similar increase in tyrosine phosphorylation was confirmed in ScGT1a (Fig. 3C), showing a 39% increase in the pTyr-staining of whole cell lysate compared to GT1 cells. Fig. 3C, right panel, shows a 2.6-fold increase in the pTyr-staining of anti-pTyr-immunoprecipitates.

A possible explanation for the different sets of tyrosine phosphorylated proteins in ScGT1 compared to ScN2a, could be their different neuronal origins. The N2a cells are derived from a spontaneous neuroblastoma arising in A/J mice [21] whereas the GT1 cells originate from hypothalamic neurons, immortalized by genetically targeted tumorigenesis in transgenic mice [22]. Correspondingly, the enhanced cellular response (i.e. increased Src kinase level and protein tyrosine phosphorylation) in ScN2a compared to ScGT1 cells, despite the lower amount of PrP^{Sc} in ScN2a compared to ScGT1a/b could also be due to their different neuronal origins. However and interestingly, within the same cell type (ScGT1a/b), there is an apparent positive correlation between the amount of PrP^{Sc} and the degree of increased Src kinase level and tyrosine phosphorylation of cellular proteins (Figs. 1–3).

3.4. *Elevated tyrosine phosphorylation in scrapie-infected cells is inhibited with the selective Src kinase inhibitor, PP2*

pTyr staining of whole cell lysates and anti-pTyr immunoprecipitates were assessed after treatment of GT1/ScGT1b and N2a/ScN2a with the Src specific inhibitor PP2 for 24 h. In ScGT1b, PP2 treatment for 24 h reversed the protein tyrosine phosphorylation to the same basal level as in GT1, clearly demonstrating that the increased tyrosine phosphorylation in ScGT1b is due to increased Src kinase activity (Fig. 4A/B, left panels). A significant decrease in protein tyrosine phosphorylation was also observed in ScN2a after a 24 h-treatment with PP2 (Fig. 4A/B, right panels), although not to the same degree as in ScGT1b. This suggests that also other protein tyrosine kinases e.g. the Abl or focal adhesion kinase (FAK) families, not inhibited by PP2, are upregulated in ScN2a cells. ScN2a cells were also treated with 3 μM PP2 for 24 h, however no further decrease in protein tyrosine phosphorylation was observed compared to ScN2a treated with 1 μM PP2 (data not shown).

3.5. *Increased in vitro Src kinase activity in scrapie-infected cells – inhibited by PP2*

To verify the increased Src kinase activity in ScGT1b and ScN2a as detected by clone 28-immunoreactivity, an in vitro kinase assay was performed using Src-immunoprecipitates. The N-16 antibody was used to immunoprecipitate Src, as binding of Src to the other available antibody may per se stimulate Src activity as reviewed in [23]. Densitometric measurements of the autoradiography of [^{32}P]-labeled Src substrate, enolase, showed a 71 ± 2.3% and a 72 ± 4.0% increased kinase activity in ScGT1b and ScN2a, respectively, compared to uninfected controls (Fig. 5). Addition of PP2 to the in vitro kinase reaction mix completely inhibited phosphorylation of enolase, again confirming the involvement of increased Src kinase activity in ScGT1b and ScN2a. The clone 28-immunoreactivity of N-16-IPs shows a 35 ± 5.3% and a 68 ± 7.5% increase in ScGT1b and ScN2a, respectively, compared to non-infected cells (data not shown). This increase corresponds to the increase in total Src kinase protein levels (clone 327-immunoreactivity of the same N-16-IPs) in both scrapie-infected cell lines compared to non-infected. The higher increase in Src kinase activity in ScGT1b observed by the in vitro kinase assay compared to the increase observed with clone 28 staining may

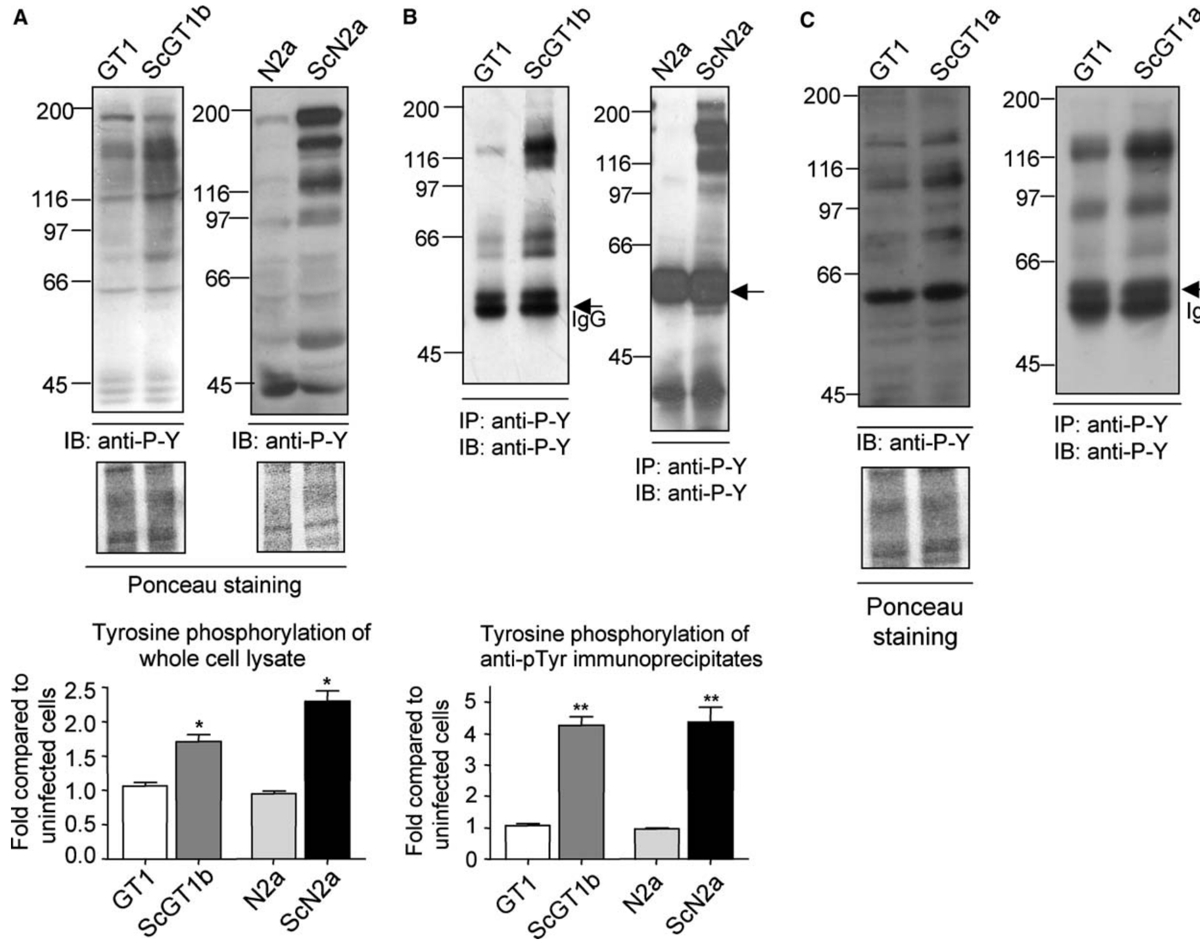

Fig. 3. Increased protein tyrosine phosphorylation in scrapie-infected cells. (A) Anti-pTyr staining of whole cell lysates of GT1, ScGT1b, N2a and ScN2a (20 μg protein/lane). Ponceau staining of the blot confirms equal amount of loaded proteins. Diagram beneath shows densitometric analysis of anti-pTyr staining. (B) 1 mg of protein from GT1, ScGT1b, N2a and ScN2a was immunoprecipitated with anti-pTyr antibody and immunoblotted with the same antibody. IgG-staining is indicated and serves to confirm equal amount of added antibody. The diagram shows densitometric analysis of the anti-pTyr staining, $^{*} = P < 0.05$, $^{**} = P < 0.01$, Student's *t*-test, $n = 3$. (C) pTyr-staining of whole cell lysate (20 μg protein) from GT1 and ScGT1a (left panel) and pTyr staining of 1 mg of proteins from GT1 and ScGT1a immunoprecipitated with anti-pTyr antibody (right panel).

be explained by the unability of clone 28 mAb to discriminate between phospho-Y416 (active) and Y416 (less active), whereas the phospho-Y416 Src is mainly detected by the in vitro kinase assay. This suggests that the ratio of phospho-Y416 in ScGT1b and GT1 is larger than 1.

4. Discussion

This report shows, in three independently prion-infected cell lines: ScN2a, ScGT1a and ScGT1b – a significant increase in active Src-kinase protein levels. A similar finding was recently reported by R. Nixon, showing an elevation of Src-family kinases in scrapie-infected brains and in ScN2a [24]. We also show, for the first time, that the increased Src-kinase protein levels in ScGT1a/b and ScN2a cells is associated with a significant elevation in protein tyrosine phosphorylation, which was efficiently blunted in the presence of the Src-kinase inhibitor PP2.

While considerable knowledge has accumulated on the regulation of Src kinase activity, virtually nothing is known about the transcriptional regulation of this kinase. However, besides a transcriptional and/or translational regulation, recent evidence suggests that Src-kinases are subject to a ubiquitin-dependent degradation, involving the Src-substrate c-Cbl E3 ubiquitin ligase [25,26]. Ubiquitin-dependent proteolysis is also responsible for the elimination of misfolded proteins, including PrP^{Sc} in prion-infected neurons [27], and in scrapie-infected mouse brains the level of ubiquitinated proteins was significantly increased, concurrent with a decline in the proteasomal endopeptidase activity [28]. This suggests that the accumulation of PrP^{Sc} in infected cells overwhelms the ubiquitin-proteasome system and therefore, if this system is deregulated in scrapie-infected cells, the result may well be elevated levels of activated Src. Further studies are required to establish why the steady state level of Src is increased in scrapie-infected cells.

The important changes in protein tyrosine phosphorylation observed in ScGT1a/b and ScN2a cells certainly have important consequences on cell signalling, synaptic function and viability. Interestingly, tau and several other neuronal proteins were shown to be tyrosine phosphorylated in response to the amyloid β peptide in a Src-dependent manner [29].

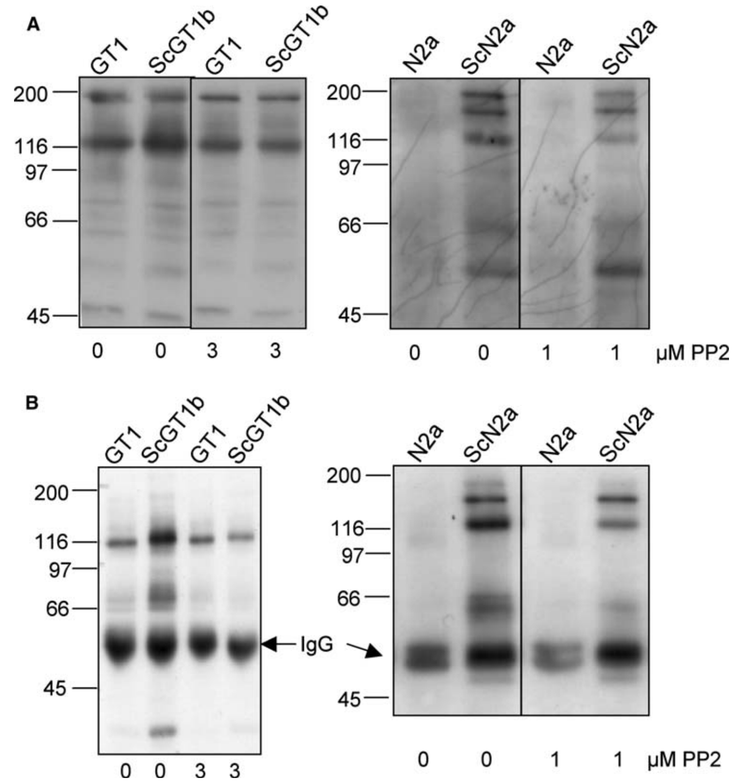

Fig. 4. The Src-kinase specific inhibitor PP2 effectively inhibits scrapie-induced protein tyrosine phosphorylation. (A) Anti-pTyr staining of whole cell lysates (20 μg protein/lane) of GT1, ScGT1b, N2a and ScN2a untreated or treated with 1 or 3 μM PP2 for 24 h. (B) Cells were untreated or treated with 1 or 3 μM PP2 for 24 h, as indicated. 1 mg of cellular proteins was immunoprecipitated with anti-pTyr antibody and immunoblotted with anti-pTyr antibody. IgG-staining confirms equal amount of added antibody.

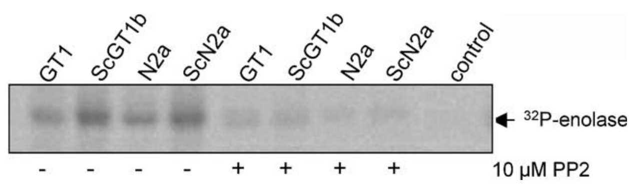

Fig. 5. In vitro Src kinase assay. The exogenous Src kinase specific substrate enolase was incubated with immunoprecipitated Src kinase (using N-16) and [γ-^{32}P]ATP in a reaction buffer, 30 min at 30 °C, and thereafter separated by 10% SDS–PAGE. The in vitro Src kinase assay was performed in the absence or presence of 10 μM PP2, which was added 30 min before [γ-^{32}P]ATP. The gel was dried and exposed to a X-ray film for 18 h. Autoradiography revealed phosphorylated enolase at 47 kDa. Densitometric analysis was performed on a PC using the program Image Gauge, v. 3.46. The control contained the same reaction components except immunoprecipitated Src kinase.

In both ScGT1b and ScN2a cells, the increased tyrosine phosphorylation was efficiently reversed by a Src-kinase inhibitor – completely in ScGT1b cells, whereas only partly in ScN2a. This suggests that also other Src-independent tyrosine kinase activities are upregulated in ScN2a cells. Of note, the tyrosine kinase inhibitor STI571 induces cellular clearance of PrP^{Sc} in prion-infected cells by activation of lysosomal degradation of PrP^{Sc} [30]. Thus, a possible scenario is that PrP^{Sc} induces an increased tyrosine kinase activity, which slow down cellular clearance of PrP^{Sc} and thereby facilitates PrP^{Sc} replication.

In conclusion, the upregulated Src-kinase activity and consequent increase in protein tyrosine phosphorylation in prion-infected neurons may cause or contribute to synaptic disorganization and loss, which is a fundamental and constant feature of prion diseases.

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